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Biochimica et Biophysica Acta 1410 (1999) 248–261

BIOCHIMICA ET BIOPHYSICA ACTA

**BBA**

# Proton to electron stoichiometry in electron transport of spinach thylakoids

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Received 23 October 1998; accepted 6 January 1999

## Abstract

According to the concept of the Q-cycle, the  $H^+/e^-$  ratio of the electron transport chain of thylakoids can be raised from 2 to 3 by means of the rereduction of plastoquinone across the cytochrome  $b_6f$  complex. In order to investigate the  $H^+/e^-$  ratio we compared stationary rates of electron transport and proton translocation in spinach thylakoids both in the presence of the artificial electron acceptor ferricyanide and in the presence of the natural acceptor system ferredoxin+NADP. The results may be summarised as follows: (1) a variability of the  $H^+/e^-$  ratio occurs with either acceptor.  $H^+/e^-$  ratios of 3 (or even higher in the case of the natural acceptor system, see below) are decreased towards 2 if strong light intensity and low membrane permeability are employed. Mechanistically this could be explained by proton channels connecting the plastoquinol binding site alternatively to the lumenal or stromal side of the cytochrome  $b_6f$  complex, giving rise to a proton slip reaction at high transmembrane  $\Delta pH$ . In this slip reaction protons are deposited on the stromal instead of the lumenal side. In addition to the pH effect there seems to be a contribution of the redox state of the plastoquinone pool to the control of proton translocation; switching over to stromal proton deposition is favoured when the reduced state of plastoquinone becomes dominant. (2) In the presence of NADP a competition of both NADP and oxygen for the electrons supplied by photosystem I takes place, inducing a general increase of the  $H^+/e^-$  ratios above the values obtained with ferricyanide. The implications with respect to the adjustment of a proper ATP/NADPH ratio for  $CO_2$  reduction are discussed. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Cytochrome  $b_6f$  complex;  $H^+/e^-$  ratio; Photosynthetic electron transport; Plastoquinone pool; Proton channel; Proton translocation; Q-cycle

Abbreviations:  $c(H^+)_i$ , internal (lumenal) proton concentration;  $c(X)$ , concentration of X; cyt, cytochrome; Fd, ferredoxin; Fecy, 'ferricyanide',  $K_3[Fe(CN)_6]$ ;  $J$ , rate of proton flux across the thylakoid membrane; PS I, photosystem I; PS II, photosystem II;  $r_{e/ATP}$ , overall electron to ATP ( $e^-/ATP$ ) ratio;  $r_{H/ATP}$ , proton to ATP ( $H^+/ATP$ ) ratio at the ATP synthase;  $r_{H/e}$ , overall proton to electron ( $H^+/e^-$ ) ratio;  $v_e$ , rate of linear electron flow;  $v_{ATP}$ , rate of ATP synthesis

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## 1. Introduction

Photosynthetic electron transport from water to the terminal acceptor is mediated by three protein complexes embedded in the thylakoid membrane: PS II, the cytochrome  $b_6f$  complex, and PS I. In addition, there are two soluble electron carriers, plastoquinone and plastocyanin, which connect PS II with cyt  $b_6f$  and cyt  $b_6f$  with PS I, respectively. For

every electron transported along the linear path two protons are injected into the thylakoid lumen, one of them arising from the oxidation of water at PS II, the other one from the oxidation of plastoquinol at cyt  $b_6f$ . According to the concept of the Q-cycle a third proton may be translocated by means of the rereduction of plastoquinone across the cytochrome  $b_6f$  complex [1,2]. The reductant for this reaction is semiquinone being formed at the  $Q_o$ -site on the luminal side of cyt  $b_6f$ . The proton gradient is used for the synthesis of ATP from ADP and  $P_i$  by means of the  $H^+$ -ATP synthase, which is the fourth major protein complex of the thylakoid membrane.

The cytochrome  $b_6f$  complex (for review see [3–7]), which is homologous to the cytochrome  $bc_1$  complexes of bacteria and mitochondria [8,9], occupies a central position in the electron transport chain [10]. The complex is formed by the four large subunits cyt  $f$ , cyt  $b_6$ , Rieske iron-sulphur protein and subunit IV, which does not participate in electron transfer reactions. Additional small subunits have been reported [11]. Details of the dual function of cyt  $b_6f$  as a plastoquinol oxidising and plastoquinone reducing enzyme are still under debate.

The  $H^+/e^-$  ratio is defined as the number of protons which are translocated across the thylakoid membrane from outside to inside per each electron passing the photosynthetic electron transport chain from water to the terminal acceptor. Most experiments on the  $H^+/e^-$  stoichiometry have been done in isolated thylakoids in the presence of the artificial electron acceptors ferricyanide or methyl viologen. These experiments verify the number of  $H^+$  coupling sites along the linear path of the electron transport system and provide information on Q-cycle activity. Initially a  $H^+/e^-$  ratio of 2 was reported for conditions of both saturating continuous light [12,13] and single-turnover flashes [14–17]. For mechanistic reasons this value of 2 seemed plausible. However, later on the picture changed and  $H^+/e^-$  ratios above 3 were observed in several laboratories. In continuous light a variation of the  $H^+/e^-$  ratio in dependence on the light intensity was reported. Lowering the light intensity to subsaturating intensities gave rise to an increased  $H^+/e^-$  ratio up to 3 [18–20]. With respect to single-turnover flashes increased  $H^+/e^-$  ratios up to 3 were observed if the time interval between the flashes was set to a value near 0.3 s [21–24]. The low

values of 2 turned out to be an artefact due to an extreme wide flash spacing with dark times above 1 s [25]. Since these new results gave strong support to the model of the Q-cycle, the notion of a fixed  $H^+/e^-$  ratio of 3 gained acceptance [26]. The concept of an obligatory Q-cycle is in fact providing an elegant framework for understanding the redox reactions in the cytochrome  $bc_1$  complex as well as in the cytochrome  $b_6f$  complex [5–7,27–29]. Therefore, in recent years this concept has emerged as the consensus view shared by most workers in the field. Although  $H^+/e^-$  ratios in vivo are difficult to obtain, there is evidence for a fixed  $H^+/e^-$  ratio of 3 also during photosynthesis under in vivo conditions [26,30,31].

The physiological aspect of the  $H^+/e^-$  ratio is concerned with the ATP/NADPH ratio generated by the light reactions. Since the  $H^+$ /ATP coupling ratio at the ATP synthase equals 4 [32–34] and the Calvin cycle needs an ATP/NADPH ratio of 1.5 in order to proceed optimally, the required  $H^+/e^-$  ratio under physiological conditions is at least 3.  $H^+/e^-$  ratios above 3 are possible by proton contributions from cyclic electron pathways such as the ferredoxin cycle [35,36] or the oxygen consuming Mehler reaction [37,38], both of which may occur in the presence of the natural terminal electron acceptor NADP.

Here we present investigations on the operation of the photosynthetic electron transport chain under steady-state conditions with both Fecy or NADP being present. In either case the variation of the  $H^+/e^-$  ratio in dependence on light intensity and membrane permeability is demonstrated. This effect on an energetic decoupling calls for a modification of the conventional Q-cycle concept. Moreover, in the presence of NADP a general increase of the  $H^+/e^-$  ratios above the values obtained in the presence of Fecy is observed, indicating the occurrence of cyclic electron pathways. The mechanistic and physiological implications of these results will be discussed in detail.

## 2. Materials and methods

### 2.1. Materials

Spinach thylakoids were prepared according to a protocol based on [39] and routinely frozen and

stored in liquid nitrogen until use (3 mM chlorophyll in the presence of 0.1 M sorbitol and 30% ethylene glycol). No difference in activity to fresh material could be detected. The experiments were performed with suspensions of thylakoids equivalent to 10  $\mu$ M chlorophyll in an optical cuvette (cross-section  $15 \times 15$  mm) at 20°C and pH  $8.00 \pm 0.05$ . The initial pH never changed for more than 0.05 units during each experiment, ensuring an unchanged buffer capacity. Actinic DC light (Schott RG 630) was given from two opposite sides.

The reaction medium contained in a volume of 4 ml 50 mM KCl, 0.3 mM Tricine buffer and, as electron acceptor, 0.2 mM ferricyanide or 0.15 mM NADP. Since ferredoxin is lost during the thylakoid preparation, in the case of NADP also 3.3  $\mu$ M Fd was added. Substrates for phosphorylation were 1 mM ADP, 3 mM  $\text{MgCl}_2$  and 1 mM phosphate. For pseudophosphorylation [40] phosphate was replaced by variable amounts of arsenate. In all experiments without phosphorylation or pseudophosphorylation 25  $\mu$ M ADP was present for suppression of the slip reaction of the  $\text{H}^+$ -ATP synthase [41]. 20  $\mu$ M phenol red or cresol red was added for optical detection of external pH changes and 4  $\mu$ M *N*-(1-naphthyl)-ethylenediamine (NED) for optical detection of internal pH. In all experiments, except for the data in Fig. 2 below, 0.4 mM imidazole was added, ensuring a facilitated  $\text{H}^+$ -efflux measurement as described in [32] (a secondary effect of imidazole is a slight enhancement of membrane permeability for protons). 2  $\mu$ M of the  $\text{K}^+$  ionophore nonactin [42] was added for suppression of the light-off transmembrane diffusion potential (see Section 2.2). Other additions are stated in the figure legends.

## 2.2. Methods

Rates of linear electron flow, proton flow and ATP synthesis were all measured by means of the coupled pH changes in the medium. These were monitored by change of absorbance of the pH indicators phenol red or cresol red (phenol red is used together with Fecy, while in the presence of Fd/NADP it is replaced by cresol red). A dual wavelength measurement at 575 and 557 nm enabled the exclusion of contributions from light scattering changes. The dual wavelength principle is realised by means of

two groups of LEDs, pulsed at a frequency of 8 kHz, and a lock-in amplifier. The light of the LEDs is guided by glass fibres.

The rate of electron flow,  $v_e$ , was obtained from the continuous pH decrease in the light due to net proton production during water oxidation according to [43] (two  $\text{H}^+$  liberated per two electrons transported in the case of ferricyanide reduction and one  $\text{H}^+$  in the case of NADP reduction). The rate of proton flow,  $J$ , was obtained from the transient pH decrease produced by proton efflux in the dark [32,44]. The initial rate in the dark equals the rate of proton translocation under steady-state conditions in the light, if the light-off diffusion potential (positive outside) is suppressed by 2  $\mu$ M nonactin (alternatively 0.5  $\mu$ M valinomycin). The omission of ionophore would give rise to a delayed discharge of the  $\text{H}^+$  gradient [13]. The rate of ATP synthesis,  $v_{\text{ATP}}$ , was obtained from the continuous pH increase in the light according to [32] (0.94  $\text{H}^+$  consumed per ATP formed at pH 8.0 and pMg 2.5). In this case the electron cofactor ferricyanide was replaced by 100  $\mu$ M methyl viologen which guarantees that the proton release due to water oxidation is totally balanced by proton uptake during the reduction of methyl viologen and subsequently oxygen.

Internal pH was obtained from the fluorescence quenching of NED according to [34] using light of 353 nm for excitation and 463 nm for detection of fluorescence, which was guided by quartz fibres dipping into the suspension from above. It should be stressed that the determination of internal pH as realised by [34] is not based on theoretical calculations dependent on a priori assumptions; the fluorescence quenching signal is calibrated by purely empirical means. Therefore, this procedure does not suffer from problems such as amine binding to the membrane surface.

## 2.3. Experimental procedure

### 2.3.1. Determination of the $\text{H}^+/\text{e}^-$ ratio

A typical pH trace from which the  $\text{H}^+/\text{e}^-$  ratio is obtained is shown in Fig. 1a. Linear electron flow,  $v_e$ , is measured from the continuous pH decrease in the light due to proton release during water oxidation. The proton flow,  $J$ , is measured from the initial pH decrease in the dark due to the efflux of protons

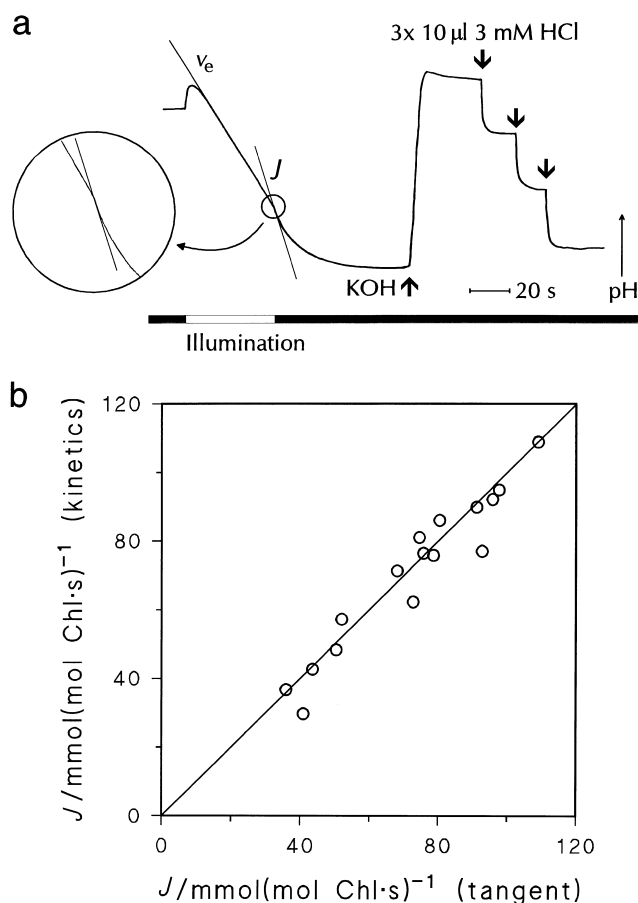


Fig. 1. (a) Time course of the external pH changes during one reaction cycle. See text for further explanations. (b) Comparison of proton efflux data obtained from identical measurements by drawing the tangent graphically (abscissa) and by kinetic analysis of proton efflux according to [32] (ordinate). Efflux rate was varied by variation of light intensity in the presence of 0.2 mM ferricyanide as electron acceptor, other conditions see Section 2.

which had been accumulated in the thylakoid lumen during illumination. Calibration with respect to external  $H^+$  turnover is achieved by injecting three times  $10 \mu\text{l}$  of 3 mM HCl into the stirred suspension at the end of each experiment (during the illumination and post-illumination phases the stirrer remains turned off). In order to perform the calibration in the relevant pH range, the pH is adjusted to its initial value by addition of KOH before HCl is added. The three consecutive calibration steps are of almost identical size which shows that the system is working sufficiently linear over the pH range of the measurement. The determination of the efflux rate is done by

drawing a tangent graphically. Alternatively, the decay of the proton gradient in the dark can be analysed by means of a kinetic equation [32]; this is done additionally for some measurements in order to check the tangent method (Fig. 1b). Both methods yield essentially the same results of the initial rate of proton efflux in the dark ( $J$ ). The  $H^+/e^-$  ratio is calculated as  $r_{H/e} = J/v_e$ . It should be noted that  $r_{H/e}$  defined this way does not necessarily reflect a mechanistic coupling ratio; it represents the total balance of proton translocation caused by both non-cyclic and cyclic electron pathways normalised to the rate of noncyclic electron transport (reduction of the terminal acceptor).

### 2.3.2. Determination of the $e^-/ATP$ ratio

The  $e^-/ATP$  ratio is obtained by the following protocol: (a) in a first experiment the net pH change due to the combined effects of  $v_e$  and  $v_{ATP}$  is measured in the presence of 1 mM ADP, 1 mM phosphate and 0.2 mM Fecy. (b) In a second measurement  $v_{ATP}$  is determined independently of  $v_e$  (Fecy being substituted by 0.1 mM methyl viologen) at the same internal pH, which is adjusted by variation of light intensity. (c)  $v_e$  is obtained from the difference of pH changes (a)–(b) and the  $e^-/ATP$  ratio is calculated as  $r_{e/ATP} = v_e/v_{ATP}$ . (d) In a third experiment the basal rate of proton efflux,  $J_b$ , is determined (ADP being reduced to  $25 \mu\text{M}$  for suppression of the slip reaction, omission of phosphate), again for the same internal pH as in (a).

## 3. Results in the presence of ferricyanide

### 3.1. Increasing light intensity lowers the $H^+/e^-$ ratio

In Fig. 2 the dependence of the rate of linear electron transport and of the  $H^+/e^-$  ratio on irradiance are shown for basal membrane permeability (i.e. lowest membrane permeability in the absence of protonophores or phosphorylation). At limiting light intensity,  $r_{H/e}$  equals 3 and declines to values near 2 at high intensities, when  $v_e$  reaches its saturation level. We would like to stress the fact that a crucial prerequisite for correct  $H^+/e^-$  measurements is the presence of a sufficient amount of a  $K^+$  ionophore like nonactin or valinomycin. These substances enable an

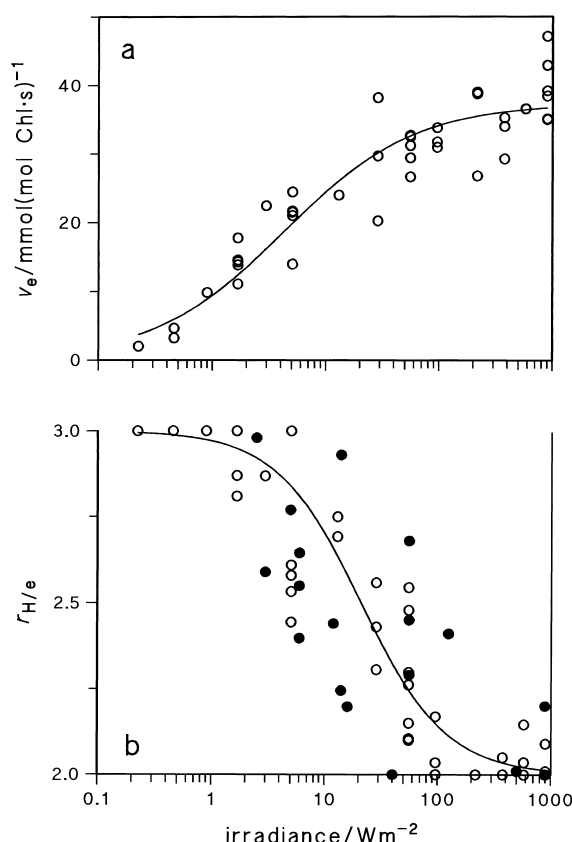


Fig. 2. Influence of irradiance on stationary rate of linear electron transport (a) and  $H^+/e^-$  ratio (b) under basal conditions (lowest membrane permeability, imidazole was omitted from the suspension medium of the thylakoids) in the presence of ferricyanide. Closed circles refer to results obtained with freshly prepared thylakoids (unpublished results by M. Rathenow, see [19] for experimental procedure). All open circle data are from the same batch of thylakoids. For closed circle data a different batch was used. In this case the absolute rates of electron transport were different, therefore the respective data are not included in (a).

accelerated  $K^+$  counterflow required for suppressing the  $H^+$  diffusion potential, which would otherwise delay the proton efflux in the dark. Suppression of the light-off diffusion potential by 2  $\mu M$  nonactin is verified directly by the analysis of the electrochromic absorbance changes occurring at 520 nm (data not shown, see [32]). In the absence of ionophores an underestimation of the  $H^+/e^-$  ratios takes place producing erroneous values of approximately 1.3 at high irradiance (data not shown). Moreover, we would like to stress the fact that the results are independent of the pretreatment of the thylakoids, which are either stored in liquid nitrogen before use (normal

case) or are used immediately after preparation (for comparison see Fig. 2b). With some of the preparations an additional effect was observed, namely a decline of the  $H^+/e^-$  ratios to values near 2 when the irradiance was reduced to very low values below 1  $W m^{-2}$  (data not shown). This effect has been noticed already in a previous study [45].

Additional experiments have been performed under pseudophosphorylating conditions in the presence of 1 mM ADP and 1 mM arsenate (Fig. 3). The produced ADP-arsenate decays immediately after release from the ATP synthase; therefore, pseudophosphorylation gives rise to increased membrane permeability by means of coupled proton flow through the ATP synthase without net consumption of ADP.

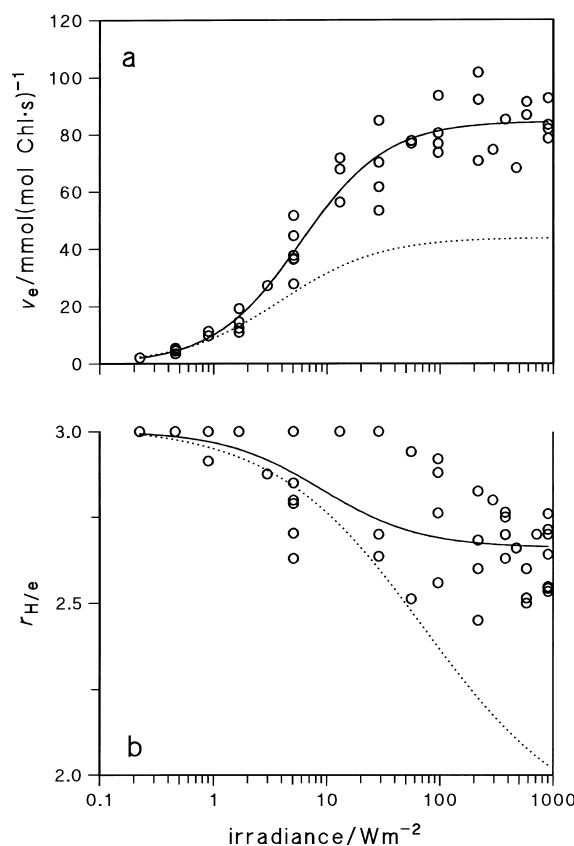


Fig. 3. Influence of irradiance on stationary rate of linear electron transport (a) and  $H^+/e^-$  ratio (b) under pseudophosphorylating conditions (1 mM ADP, 1 mM arsenate and 3 mM  $MgCl_2$ ) in the presence of ferricyanide. For use of comparison the dotted curve refers to experiments in the absence of pseudophosphorylation. All data are from the same batch of thylakoids as in Fig. 2 (open circles).

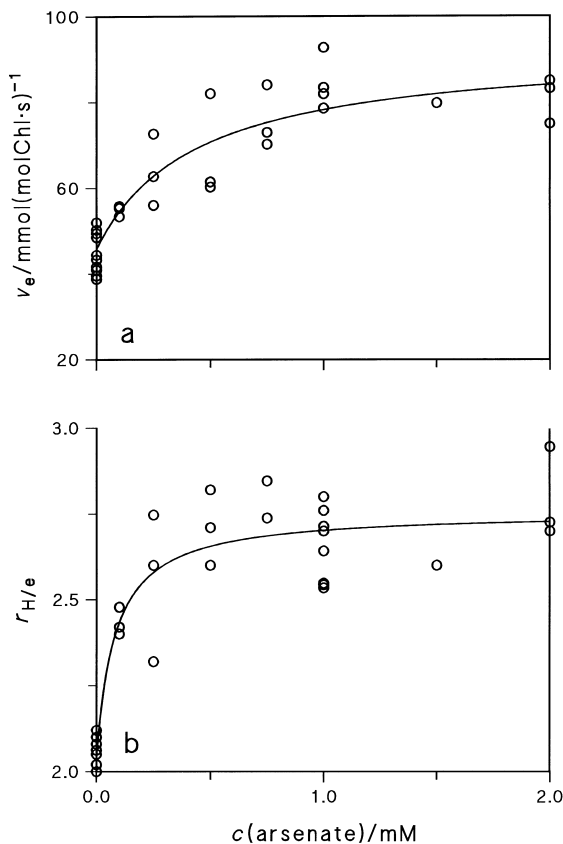


Fig. 4. Influence of the arsenate concentration on stationary rate of linear electron transport (a) and  $\text{H}^+/\text{e}^-$  ratio (b) under pseudophosphorylating conditions (1 mM ADP and 3 mM  $\text{MgCl}_2$ ) in the presence of ferricyanide at constant light intensity of  $900 \text{ W m}^{-2}$ . All data are from the same batch of thylakoids as in Fig. 2 (open circles).

This enables  $\text{H}^+/\text{e}^-$  measurements which are undisturbed by additional outer phase pH changes due to ATP synthesis. Dissipation of the transmembrane  $\Delta\text{pH}$  by means of coupled proton flow through the ATP synthase induces an increase in the rate of linear electron transport. Compared to basal conditions, the value at light saturation is approximately doubled. This effect occurs because the rate limiting step of the electron transport chain, namely the oxidation of plastoquinol at the  $\text{cyt } b_6f$  complex, is under control of the internal pH and therefore affected by membrane permeability. Under pseudophosphorylating conditions, there is also a decline of  $r_{\text{H}/\text{e}}$  from 3.0 at limiting light to 2.7 at maximum irradiance of  $900 \text{ W m}^{-2}$ . However, this effect (Fig. 3b) is much smaller in comparison to the strong decline of  $r_{\text{H}/\text{e}}$  under basal conditions (Fig. 2b).

### 3.2. Increasing membrane permeability increases the $\text{H}^+/\text{e}^-$ ratio

Comparison of Figs. 2b and 3b shows a marked increase of  $r_{\text{H}/\text{e}}$  at high light intensity when membrane permeability is increased by pseudophosphorylation. This effect is investigated in more detail in Fig. 4. At a constant irradiance of  $900 \text{ W m}^{-2}$ , membrane permeability is varied by addition of variable concentrations of arsenate (at a fixed concentration of 1 mM ADP). Fig. 4a shows the increase of  $v_e$  with increasing membrane permeability. The corresponding  $\text{H}^+/\text{e}^-$  ratios are shown in Fig. 4b. The starting value of 2.1 is lifted to 2.7. Instead of using pseudophosphorylation, the proton permeability of the thylakoid membrane can also be increased by the use of

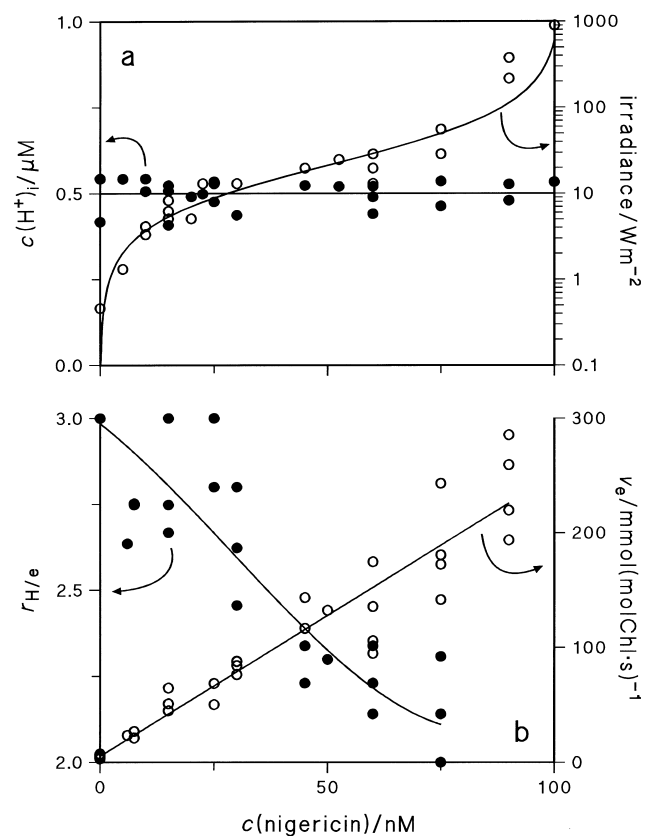


Fig. 5. Influence of nigericin concentration on the required irradiance for clamping the initial internal proton concentration of  $0.5 \mu\text{M}$  (a) and concomitant  $\text{H}^+/\text{e}^-$  ratio and rate of linear electron transport (b) in the presence of ferricyanide. All data are from the same batch of thylakoids. Reliable determination of  $\text{H}^+/\text{e}^-$  ratios above 75 nM nigericin is not possible due to the vanishing proton gradient under these conditions.

artificial protonophores. We tested the substances nigericin, monensin and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and the results are the same as in the case of pseudophosphorylation: increase of membrane permeability results in an increased rate of linear electron transport with a concomitant increase of the  $H^+/e^-$  ratio (data not shown).

### 3.3. There is no definite correlation between intrathylakoid pH and the $H^+/e^-$ ratio

The results of Figs. 2–4 suggest a regulation of  $r_{H/e}$  by the internal pH of the thylakoids: increasing light intensity gives rise to an increased pH gradient, while increased membrane permeability dissipates part of this gradient. This concept includes the prediction that the  $H^+/e^-$  ratio should depend on the value of the internal proton concentration,  $c(H^+)_i$ , only, irrespective of the way of adjustment of  $c(H^+)_i$ . This prediction is tested in Fig. 5. Starting at a low light intensity, the membrane permeability is increased by rising concentrations of the protonophore nigericin. At the same time the irradiance is increased in order to keep the internal proton concentration constant. Fig. 5a shows the irradiance which is necessary to maintain the initial value of  $c(H^+)_i = 0.5 \mu M$  in the presence of the corresponding

concentration of nigericin. Fig. 5b shows the rate of linear electron transport in dependence on the nigericin concentration and the resulting  $H^+/e^-$  ratios. Obviously, there is no constant  $H^+/e^-$  ratio as would be expected if it were a function of internal pH only. The stimulating effect of increasing membrane permeability in the presence of increasing nigericin concentrations is overcompensated by the decrease due to rising irradiances, therefore the net effect is a decline of  $r_{H/e}$ .

### 3.4. $H^+/e^-$ ratio from the $e^-/ATP$ stoichiometry

The  $e^-/ATP$  stoichiometry,  $r_{e/ATP}$ , can be obtained as the ratio  $v_e/v_{ATP}$ . This stoichiometry is related to the  $H^+/e^-$  ratio,  $r_{H/e}$ , via

$$r_{e/ATP} = v_e/v_{ATP} = r_{H/ATP}/r_{H/e}, \quad (1)$$

with  $r_{H/ATP}$  being the  $H^+/ATP$  ratio at the ATP synthase. Therefore, Eq. 1 enables in principle the determination of  $r_{H/e}$  ( $r_{H/ATP}$  given) from  $e^-/ATP$  measurements without an involvement of efflux measurements. However, this equation holds only in the absence of basal proton efflux. Since the inherent leakiness of the membrane has to be taken into account, Eq. 1 is modified to

$$r_{e/ATP} = \frac{r_{H/ATP} + J_b/v_{ATP}}{r_{H/e}}, \quad (2)$$

with  $J_b$  representing the basal portion of total proton efflux.  $J_b$  has to be determined independently by means of efflux measurements as done in the previous sections. A determination of  $r_{H/e}$  according to the rationale of Eq. 2 therefore comprises three measurements, all of which must refer to the same internal pH (adjusted by light intensity): (a) determination of  $v_e$ , (b) determination of  $v_{ATP}$ , and (c) determination of  $J_b$ .  $r_{H/ATP}$  is taken as being known, having a value of 4 [32–34]. Table 1 shows the results for sets of experiments where the initial light intensity (in the measurement of  $v_e$ ) was 60 or 900  $W m^{-2}$ . The averaged  $r_{H/e}$  is in both cases 2.6, which is close to the value of 2.7 obtained by the efflux method under pseudophosphorylating conditions as described above (compare Fig. 3).

Table 1

Determination of the  $H^+/e^-$  ratio from measurements of  $v_e$ ,  $v_{ATP}$  and  $J_b$

Irradiance ( $W m^{-2}$ )	$v_e$	$v_{ATP}$	$J_b$	$r_{e/ATP}$	$r_{H/e}$
60	62.3	29.9	35.8	2.1	2.5
60	70.5	35.1	42.8	2.0	2.6
60	66.7	25.6	76.7	2.6	2.7
Mean value					2.6
900	62.0	29.6	34.7	2.1	2.5
900	77.6	36.9	44.2	2.1	2.5
900	79.7	35.4	70.1	2.3	2.7
900	56.3	21.6	70.9	2.6	2.8
Mean value					2.6

All rates in units of  $mmol (molChl-s)^{-1}$ .

Different rows refer to experiments done with different batches of thylakoids according to the strategy as described in the text. For experimental details see Section 2.3.2. The  $H^+/e^-$  ratio has been calculated on the basis of Eq. 2 taking into account  $r_{H/ATP} = 4$ .

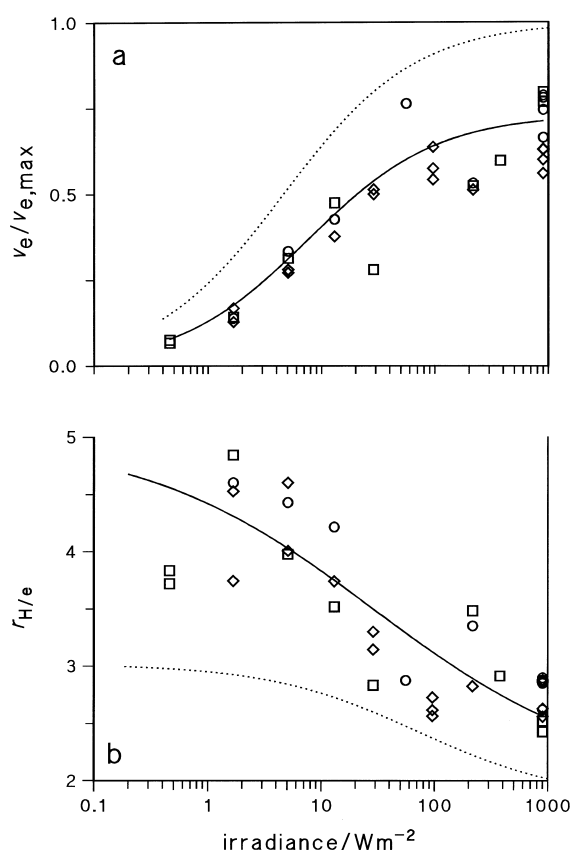


Fig. 6. Influence of irradiance on the relative stationary rate of linear electron transport (a) and on the  $H^+/e^-$  ratio (b) with ferredoxin and NADP as electron acceptor. The rates are normalised to the maximum value in the presence of ferricyanide. Different symbols represent different batches of thylakoids. The dotted curves refer to the Fecy data for comparison.

#### 4. Results in the presence of ferredoxin and NADP

##### 4.1. Similar effects of increasing light intensity as with Fecy

Fig. 6a shows the dependence of the rate of linear electron transport on irradiance under nearly basal conditions (slightly increased membrane permeability due to the presence of 0.4 mM imidazole) if Fecy is replaced by Fd/NADP. The values of  $v_e$  are normalised with the maximum rate in the presence of Fecy ( $v_{e,max}$ ) under identical conditions as the point of reference. The  $v_e$  at light saturation is only approximately 70% of the saturation rate of Fecy reduction.

Fig. 6b shows the corresponding  $H^+/e^-$  ratios. The general picture is the same as in Fig. 2b, where Fecy was present:  $r_{H/e}$  starts with high values for limiting

light intensities and is clearly decreased at high irradiances. However, the values in the presence of Fd/NADP are significantly higher at all irradiances. The occurrence of  $H^+/e^-$  ratios far above 3 is an indication of contributions by the ferredoxin cycle [35,36] and/or the Mehler reaction [37,38] to the total proton translocation balance. But even despite such additional proton translocating reactions contributing to the overall  $r_{H/e}$ , there still is the decline of  $r_{H/e}$  below 3 at high irradiances, as in the presence of Fecy.

##### 4.2. Increasing membrane permeability again increases the $H^+/e^-$ ratio

The experiment of Fig. 4 was repeated in the presence of Fd/NADP. Arsenate was added at increasing

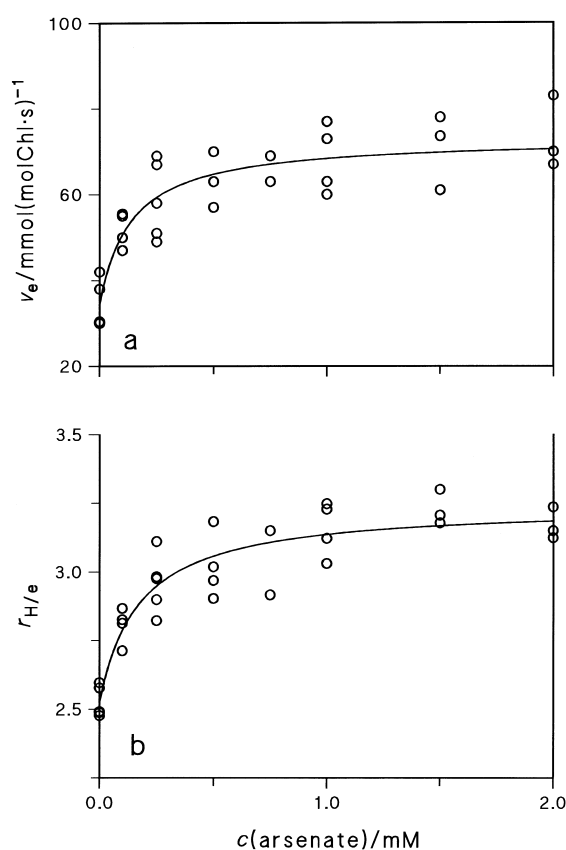


Fig. 7. Influence of the arsenate concentration on stationary rate of linear electron transport (a) and  $H^+/e^-$  ratio (b) under pseudophosphorylating conditions (1 mM ADP and 3 mM  $MgCl_2$ ) in the presence of ferredoxin and NADP at constant light intensity of  $900 W m^{-2}$ . All data are from the same batch of thylakoids as in Fig. 2 (open circles).



concentrations in the presence of 1 mM ADP and at constant irradiance ( $900 \text{ W m}^{-2}$ ), giving rise to increasing rates of pseudophosphorylation with the consequence of a corresponding increase of the rates of coupled proton flow and also electron flow (Fig. 7).  $v_e$  at arsenate saturation is doubled when compared to conditions without pseudophosphorylation (Fig. 7a). The corresponding  $\text{H}^+/\text{e}^-$  ratios increase from 2.5 to 3.2 (Fig. 7b).

## 5. Discussion

### 5.1. Reliability of the $\text{H}^+$ efflux method

The light-off pH decrease in the medium signals the breakdown of the proton gradient across the thylakoid membrane; the rate of the initial pH change gives information on the rate of the transmembrane proton efflux, which is totally balanced by light-induced proton influx during steady-state conditions. This is the rationale for the  $\text{H}^+$  efflux measurement used in this work. Its realisation requires five crucial prerequisites: (1) the light-off diffusion potential caused by electrically uncompensated proton efflux must be completely suppressed by sufficient amounts of  $\text{K}^+$  ionophores such as non-actin or valinomycin. This has been verified directly by monitoring the electrochromic absorbance changes at 520 nm. (2) The pH changes have to be recorded without delay. This has been realised by optical detection. The adequacy of the response time is demonstrated by the calibration steps in Fig. 1, which are evoking an almost immediate response (note that the total time of the calibration steps not only includes the response time of the system, but also the time for adding HCl and the subsequent mixing process in the cuvette). (3) The optical pH signal has to be free of disturbances from light scattering changes. This has been achieved by application of the method of difference spectroscopy. (4) The vertical resolution of the system should be sufficiently high for a reliable determination of the pH changes after the illumination period. For this reason routinely 0.4 mM imidazole were added resulting in a 5-fold expansion of the external pH signal [32]. (5) A trustworthy procedure of selecting the initial part of the pH trace which is used for deter-

mination of the rate of proton efflux should be available. For this reason the simple tangent method has been checked by a fitting procedure of the pH signal according to [32]. The reliability of the  $\text{H}^+$  efflux method has convincingly been demonstrated already in earlier experiments on the determination of the  $\text{H}^+/\text{ATP}$  ratio [32]. Moreover, critical results of  $\text{H}^+/\text{e}^-$  ratios below 3 obtained in this work have been verified independently by the method of  $\text{H}^+/\text{e}^-$  determination from  $\text{e}^-/\text{ATP}$  measurements.

### 5.2. Variability of the $\text{H}^+/\text{e}^-$ ratio in the presence of ferricyanide

A crucial result is the definite change of the  $\text{H}^+/\text{e}^-$  ratio within the span between 2 and 3 which is evoked by definite changes of the experimental conditions. The  $\text{H}^+/\text{e}^-$  ratio is 3 when actinic light of low intensity is employed. This value declines towards 2 when the light intensity is increased strongly (Figs. 2 and 3). These low values of  $r_{\text{H}/\text{e}}$  induced by very high light intensities are lifted again if the membrane permeability is increased (Figs. 3 and 4). The switching off and on of the third proton points to a control of the  $\text{H}^+$  translocation across the *cyt  $b_6f$*  complex. The seemingly plausible assumption of an exclusive control by the internal pH can be ruled out (Fig. 5). Rather, the control probably depends on both the redox state of plastoquinone and the internal pH, as will be discussed in Section 5.4.

Comparing the data presented here with results from the literature, a distinction has to be made between continuous light and flash-light experiments. With respect to continuous light excitation we confirm and extend results described earlier [18–20]. However, Rich [27] has attributed the decline of the  $\text{H}^+/\text{e}^-$  ratio at high light intensities to the inappropriate use of Fecy as an electron acceptor, which was assumed to cause an overestimation of linear electron transport by capturing electrons from sites other than PS I. This was concluded from the occurrence of a DCMU-insensitive electron transport at high light intensities. In our thylakoid preparations it was possible to suppress the linear electron transport completely with DCMU at concentrations of  $1 \mu\text{M}$  or higher and we therefore are convinced that an overestimation of linear electron transport is not the cause of  $\text{H}^+/\text{e}^-$  ratios smaller than 3. Moreover,

$H^+/e^-$  ratios significantly below 3 at high light intensities are observed also in the presence of Fd/NADP (see Section 5.3). On the other hand, Kobayashi et al. [30] found that  $r_{H/e}$  in the presence of Fecy always was about 2, even under conditions of increased membrane permeability. This result from [30] is at discrepancy with our own experiments. However, the experimental conditions are difficult to compare, in particular because the measurements in [30] were done without ionophore, therefore the results on transmembrane proton flow may be distorted by diffusion potentials.

In the case of single-turnover flashes at a repetition rate of 3 Hz a  $H^+/e^-$  ratio of 3 has become generally accepted [21–25]. Such conditions are comparable to those of low intensity continuous illumination ( $3 \text{ W m}^{-2}$ ), which are in fact yielding the same  $H^+/e^-$  ratio of 3. Increase of the flash repetition rate corresponds to the increase of the intensity in the case of continuous illumination. Hangarter et al. [24] reported a decrease of the  $H^+/e^-$  ratio from 2.7 down to 1.6 as a consequence of increasing the repetition rate from 3 to 10 Hz. However, this effect was shown to be flash number dependent. The decreased  $H^+/e^-$  ratio was evident only after 50 flashes, which also was the number of flashes required for reaching the steady-state level of proton uptake into the lumen. This behaviour nicely reflects the influence of the internal pH on the  $H^+/e^-$  ratio as demonstrated in the present paper. For a *decrease* of the flash repetition rate to values *below* 3 Hz also a decline of the  $H^+/e^-$  ratio to values near 2 has been reported [25]. The authors attributed this effect to a dissipative side reaction. Such conditions are comparable to those of extremely low intensity continuous illumination (below  $1 \text{ W m}^{-2}$ ). A corresponding decline of the  $H^+/e^-$  ratio has been observed previously [45], but it is observed only occasionally with the preparations used in this work.

There is evidence for an invariable  $H^+/e^-$  ratio of three *in vivo* [26,30,31]. This is not necessarily in contradiction to the results presented here, since under *in vivo* conditions the influence of phosphorylation has to be taken into account; proton efflux through the  $H^+$ -ATP synthase results in a significantly smaller  $\Delta\text{pH}$  as compared to basal conditions. In intact leaves  $\Delta\text{pH}$  was estimated to be in the range of 2.5 units at saturating light [46], while it will reach

the excessively large value of 3.5 units when high light intensity is applied at basal membrane permeability. Note that the  $H^+$ -ATP synthase *in vivo* is operating in the reduced state, which has a higher activity than the oxidised form of the enzyme. Given the strong increase of  $r_{H/e}$  in the presence of pseudophosphorylation even under conditions of oxidised  $H^+$ -ATP synthase (Fig. 3), we conclude that our results in the presence of Fecy are compatible with  $r_{H/e} = 3$  *in vivo*.

### 5.3. Comparison of ferricyanide and Fd/NADP as electron acceptors

Exchanging the electron acceptor Fecy for Fd/NADP we observe generally decreased rates of electron flow and increased  $H^+/e^-$  ratios (Fig. 6a and b). The possible reason for these shifts will be discussed below. However, despite these differences, when Fd/NADP is used as acceptor there still is at high light intensities and low membrane permeability a decline of the  $H^+/e^-$  ratio to values significantly below 3, as in the case of Fecy (Figs. 6b and 7). This behaviour excludes the possibility of a ferricyanide artefact.

The rates of linear electron transport with Fd/NADP as electron acceptor amount to about only 70% of the value in the presence of Fecy (Fig. 6). For a discussion of this discrepancy the electron flow through PS I (PS I flow) has to be taken as point of reference. One possible explanation would be that Fecy accepts electrons from sites other than PS I. In this case the observed rate of Fecy reduction would be higher than the PS I flow, giving rise to a corresponding underestimation of the  $H^+/e^-$  ratio. This explanation was favoured in [27] for the occurrence of  $r_{H/e}$  values below 3. On the other hand, it is also conceivable that the rate of NADP reduction is smaller than the PS I flow while the rate of Fecy reduction correctly reflects the PS I flow. As shown in Fig. 6, there are  $H^+/e^-$  stoichiometries far higher than 3 in the presence of Fd/NADP. According to current understanding of the photosynthetic electron transport chain, such high numbers can be only explained by additional proton translocating reactions, i.e. the ferredoxin cycle [35,36] and/or the oxygen consuming Mehler reaction [37,38]. The latter is a pseudocyclic process which increases the overall  $H^+/e^-$  stoichiometry by diverting electrons from

the terminal acceptor NADP while proton translocation remains unaffected. Both the ferredoxin cycle and the Mehler reaction would be competing with NADP for electrons from PS I, therefore there is a compelling reason to believe that the measured rate of NADP reduction underestimates the PS I flow.

In the older literature (e.g. [47]) it was proposed that ferredoxin dependent cyclic photophosphorylation is making a large contribution to the total ATP synthesis of thylakoids. However, in the last decade a different picture has emerged according to which, in particular in C3 plants such as spinach, the ferredoxin cycle mainly has a regulatory function [35,36,48]. The same physiological function of a down-regulation of PS II is also attributed to the Mehler reaction [38,49]. The controversy of the relative contributions of these two reactions seems to be not finally settled. It has been reported that under aerobic conditions the Mehler reaction is able to suppress the ferredoxin cycle completely [50]; on the other hand, it seems that the Mehler reaction alone is not sufficient to prevent photodamage at the photosystems [51]. However, the  $H^+/e^-$  ratios in excess of 3 from Fig. 6 are not affected by the ferredoxin cycle inhibitor antimycin A (data not shown). Our conclusion is that under our experimental conditions in the presence of Fd/NADP the extra proton translocation exceeding the stoichiometry of the linear chain including the Q-cycle is mostly due to the Mehler reaction.

Hall [52] presents a review of earlier results on the ATP/electron stoichiometry in the presence of Fecy and of Fd/NADP. These values can be transformed into corresponding  $H^+/e^-$  stoichiometries. In agreement with the results presented here,  $r_{H/e}$  is higher with Fd/NADP as electron acceptor than with Fecy. Kobayashi et al. [30] also found higher  $H^+/e^-$  ratios for intact chloroplasts than in the presence of ferri-cyanide.

#### 5.4. Interpretation of the variability of the $H^+/e^-$ ratio

The variation of the steady-state  $H^+/e^-$  stoichiometry, which is reported here and elsewhere [18–20], is not restricted to the photosynthetic electron transport chain. There are also reports of such a variation in mitochondrial electron transport [53,54], variable  $H^+/e^-$  ratios have been found for complex I and

complex IV [55,56] and, in particular, for the mitochondrial cytochrome  $bc_1$  complex (complex III) [57–60]. The pattern found for cyt  $bc_1$  equals the one which is reported here for cyt  $b_6f$ : high values of the proton motive force, either in the form of  $\Delta pH$  or  $\Delta\psi$ , are decreasing the  $H^+/e^-$  ratio. An inhibitory influence of  $\Delta\psi$  on the rate of deposition of the second proton has also been reported for cyt  $b_6f$  [61].

Previously we speculated on the possibility that the second electron of plastoquinol is transferred to the Rieske centre, too, following the first one [62] or that there might be a side reaction in the form of a loss of semiquinone from the cytochrome  $b_6f$  complex after the first electron transfer step [63]. Both these reactions are bypassing the Q-cycle and could therefore provide an explanation for  $H^+/e^-$  ratios below 3. However, it seems impossible to reconcile these models with other data on the cytochrome  $b_6f$  complex, from which the picture of an obligatory Q-cycle in analogy to the cyt  $bc_1$  complex has emerged [5–7,27]. In particular, the observation of a concomitant reduction of both the high-potential chain and cyt  $b_6$  after flash-induced turnover is strong evidence for an obligatory Q-cycle [64–66]. For the cyt  $bc_1$  complex the concept of the Q-cycle has been further elaborated by models for the obligatory bifurcation between the routes of the linear and non-linear electron pathways [67–69] and these models should apply to the cyt  $b_6f$  complex as well. Recent support to this obligatory bifurcation stems also from reports of a movement of the Rieske protein in cyt  $bc_1$ , giving rise to an electron shuttle between the site of quinol oxidation and cyt  $c_1$  [70,71].

However, while the electron transfer reactions in the Q-cycle obviously are strictly obligatory, there is a number of reports on the possibility to decouple the concomitant proton transfer steps: proton translocation in cyt  $bc_1$  can be decoupled from electron transfer by chemical modification of the complex with *N,N'*-dicyclohexylcarbodiimide (DCCD) [72] or with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline [73] and also by point mutations [74]. The same observation of a suppression of proton translocation with at the same time unaffected electron transfer after DCCD treatment has also been made for the cyt  $b_6f$  complex [72,75]. The existence of proton channels in cyt  $b_6f$  has been proposed more than 10 years ago [76] and it seems that such channels are

the most likely explanation of the observed proton slip reactions in cyt  $bc_1$  and cyt  $b_6f$  [28,74,75].

It would appear most plausible to assume that the blocking of luminal proton deposition, which can be artificially induced, for instance, by DCCD, can also arise from a proton backpressure at very low luminal pH. The fact that  $r_{H/e}$  is lowered by an increase of irradiance (Fig. 2) and raised by a following increase of the membrane permeability (Fig. 4) points towards a control by the intrathylakoid pH and we therefore assume that a high internal proton concentration gives rise to a complete protonation of an acceptor group, which is required for the conduction of the semiquinone proton into the lumen. In this case a slip reaction should occur which diverts the proton via a proton channel in the cyt  $b_6f$  complex towards the stromal side of the membrane. The net result is the transfer of two electrons from plastoquinol, one of them reaching the Rieske centre, the other one being transferred to cyt  $b_6$ . Of the two protons of a plastoquinol molecule, however, only one appears in the lumen while the second one is decoupled and returned into the stroma. The overall  $H^+/e^-$  ratio of the electron transport chain would be 2 under these conditions.

However, an exclusive control of  $r_{H/e}$  by internal pH would be at variance with the results of Fig. 5, where a decline of  $r_{H/e}$  is observed despite of the fact that  $c(H^+)_i$  is maintained at a constant level by the combined action of nigericin addition and irradiance increase. In this experiment the decline of  $r_{H/e}$  is accompanied by a distinct increase of  $v_e$  which gives evidence of a corresponding increase of the plastoquinol concentration. The degree of plastoquinone pool reduction seems to be the second parameter acting together with the internal pH in controlling the  $H^+/e^-$  stoichiometry. This concept is compatible with all the results of Figs. 2–5. A strongly reduced plastoquinone pool gives rise to a backpressure of electrons in the Q-cycle and consequently a reduction of the high potential heme of cyt  $b_6$ . This means an accumulation of negative charge in the low potential chain of the cyt  $b_6f$  complex, which may increase the proton affinity of the proton channel being responsible for the decoupling reaction. A plausible structural basis of such a mechanism could be an involvement of the histidine ligands of cyt  $b_6$  in the proton channel. The qualitative arguments outlined here are

supported by a corresponding quantitative modelling of the electron transport chain, which will be demonstrated elsewhere (Berry and Rumberg, manuscript in preparation).

### 5.5. Physiological significance

The findings described so far may be summarised as follows:

1. In the presence of ferricyanide under circumstances reflecting physiological conditions (low to moderately high light intensity, phosphorylation present) the  $H^+/e^-$  ratio is in the range of 3. A significant decrease of  $r_{H/e}$  towards values in the range of 2 occurs only when strong light in the absence of phosphorylation is applied.
2. In the presence of the natural electron acceptor NADP a competition of both NADP and oxygen for the electrons supplied by PS I takes place (participation of the Mehler reaction) with the consequence of a general increase of  $r_{H/e}$  above the values obtained with the artificial acceptor Fecy.

In order to assess the physiological implications of these findings, we consider the necessary  $r_{H/e}$  for balancing the production of NADPH and ATP required for  $CO_2$  reduction. Given a proper ATP/NADPH ratio of 1.5 and the  $H^+/ATP$  coupling ratio at the ATP synthase of 4 [32–34], and taking in mind that two electrons have to be transported for the production of 1 NADPH molecule,  $r_{H/e} = 3$  seems to be necessary when  $CO_2$  fixation is running. However, the basal proton flux across the thylakoid membrane has to be considered in addition to the proton flux across the ATP synthase. This portion of the total proton flux, amounting naturally 10% under conditions of saturating irradiance, will increase upon lowering the irradiance. The reason is that basal and phosphorylating  $H^+$  fluxes obey different reaction orders with respect to  $c(H^+)_i$ . Berry and Rumberg [32] found reaction orders of 0.8 and 2.6, respectively, for basal and phosphorylating flux. Therefore, if  $c(H^+)_i$  is lowered as a consequence of a decreased irradiance, the portion of the basal efflux relative to the phosphorylating efflux will be favoured. It follows that the optimal  $r_{H/e}$  needed for  $CO_2$ -reduction will be given by

$$r_{H/e} = 3(1 + J_b/J_{ATP}) = 3(1 + \alpha[c(H^+)_i]^{-1.8}), \quad (3)$$

where  $J_b$  and  $J_{ATP}$  are the basal and phosphorylating  $H^+$  fluxes and  $\alpha$  is a constant which controls the mixing of  $J_b$  and  $J_{ATP}$ . Taking into account that  $v_{ATP}$  (rate of phosphorylation) is as well as  $J_{ATP}$  proportional to the 2.6th power of  $c(H^+)_i$  and that  $v_{ATP}$  and  $v_e$  have to be proportional to each other we finally obtain for the  $r_{H/e}$  being necessary for  $CO_2$ -reduction:

$$r_{H/e} = 3(1 + \beta(v_e/v_{e,max})^{-0.69}). \quad (4)$$

Here  $\beta$  is a constant analogous to  $\alpha$  in Eq. 3. Eq. 4 shows that  $r_{H/e}$  should increase if  $v_e$  is decreased by lowering the irradiance. If we assume that under saturating irradiance ( $v_e = v_{e,max}$ ) the basal flux has a portion of 10% relative to the phosphorylating one, then  $\beta$  is determined as 0.1. In this case  $r_{H/e}$  should increase to 4 if  $v_e$  is decreased to 20% of  $v_{e,max}$  by lowering of the irradiance. These predictions are in surprising accordance to our experimental results (see Fig. 6b). The conclusion is that under physiological conditions the need of additional  $H^+$  due to the basal flux is covered by the participation of the Mehler reaction and possibly the ferredoxin cycle, in addition to the operation of the Q-cycle. The decoupling of proton translocation from electron transport in the cytochrome  $b_6f$  complex may be a dissipative reaction occurring only under extreme conditions such as water stress in combination with high irradiance.

## Acknowledgements

The authors wish to thank Dr. Martin Rathenow and Dr. Karsten Schubert for valuable preliminary studies and Monika Weiß for skilful technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 312).

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